Genetic transformation of loblolly pine using mature zygotic embryo explants by *Agrobacterium tumefaciens*

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Abstract: *Agrobacterium tumefaciens* strain LBA 4404 carrying pBI121 plasmid was used to transform mature zygotic embryos of three genotypes (E-Hb, E-Ma, and E-Mc) of loblolly pine. The results demonstrated that the expression frequency of β-glucuronidase reporter gene (GUS) varied among genotypes after mature zygotic embryos were infected with *Agrobacterium tumefaciens* cultures. The highest frequency (27.8%) of GUS expressing embryos was obtained from genotype E-Mc with mean number of 21.9 blue GUS spots per embryo. Expression of β-glucuronidase reporter gene was observed on cotyledons, hypocotyls, and radicles of transformed mature zygotic embryos, as well as on organogenic callus and regenerated shoots derived from co-cultivated mature zygotic embryos. Nineteen regenerated transgenic plants were obtained from GUS expression and kanamycin resistant calli. The presence and integration of the GUS gene was confirmed by polymerase chain reaction (PCR) and Southern blot analysis. These results suggested that an efficient *Agrobacterium tumefaciens*-mediated transformation protocol for stable integration of foreign genes into loblolly pine has been developed and that this transformation system could be useful for the future studies on transferring economically important genes to loblolly pine.

Key words: *Pinus taeda L.*; Genetic transformation; *Agrobacterium tumefaciens*; β-glucuronidase gene; Polymerase chain reaction; Southern blot

Abbreviations: BA--benzyladenine; 2,4-D—2,4-dichlorophenoxyacetic acid; GUS--β-glucuronidase gene; nptII-- phosphotransferase gene; PCR--Polymerase chain reaction

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Introduction

Genetic transformation in conifers has the potential to allow the selective improvement of individual traits in elite clones while still maintaining the existing combination of genes responsible for the superior phenotype (Charest *et al.* 1991; James *et al.* 1996; Walter *et al.* 1999). At present, although considerable research effort has been devoted to the genetic engineering of conifer species (Sederoff *et al.* 1986; Bekkauoi *et al.* 1988, 1990; Robertson *et al.* 1992; Bommineni *et al.* 1993; Shin *et al.* 1994; Klimaszewska *et al.* 1997), it has lagged behind advances made in angiosperm wood forest trees and herbaceous crops due both to economics and the recalcitrant nature of woody perennials to in vitro manipulation (Gupta *et al.* 1993; James *et al.* 1996; Wenck *et al.* 1999).

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Agrobacterium-mediated transformation has been one of the favored methods for introduction of foreign genes into higher plants. Inoculation with *Agrobacterium* resulted in tumour development in numerous coniferous species (Sederoff *et al.* 1986; Clapha *et al.* 1986; Diner *et al.* 1987; Morris et al. 1989; Stomp *et al.* 1990; Bergmann *et al.* 1992). Transient expression of GUS gene by *Agrobacterium tumefaciens* has been reported for *Pinus halepensis* Mill (Tzfira *et al.*1996) and *Pinus pinea* L. (Humara *et al.* 1999). However, transgenic plant regeneration has not obtained mainly due to the lack of a suitable regeneration procedure. Recent advances in tissue culture and transformation techniques in conifers make transgenic plantlet regeneration for coniferous species feasible. Transgenic plantlet regeneration has been obtained from *Larix deciduas* (Huang *et al.* 1991) and *Larix kaempferi* ×*L. decidua* (hybrid larch) (Levee *et al.* 1997) via cocultivation with *Agrobacterium*. More recently, transgenic plantlet regeneration in conifers has succeeded in *Picea abies* (Walter *et al.* 1999), *Picea glauca* (Ellis *et al.* 1993), *Picea mariana* (Charest *et al.* 1996), and *Pinus radiata* (Walter *et al.* 1998) via microprojectile bombardment.

Loblolly pine (*Pinus taeda* L.) is an economically

important forest tree, which is widely planted in tropical and subtropical regions. Sederoff *et al.* (1986) first reported the gene transfer by *Agrobacterium tumefaciens* in loblolly pine. Gupta *et al.* (1988) reported somatic proembryo formation and transient expression of a luciferase gene in loblolly pine protoplasts. Stomp *et al.* (1991) succeeded in transient expression from microprojectile-mediated DNA transfer in *Pinus taeda*. Wenck *et al.* (1999) reported GUS expression in cell suspension cultures of loblolly pine. Yet there is no report on *Agrobacterium tumefaciens*-mediated transformation and expression of β-glucuronidase reporter gene in mature zygotic embryo cultures and regenerated plants of loblolly pine because of the lack of tissue culture system compatible with transformation. In this study, we present the *Agrobacterium*-mediated transformation and stable expression of the β-glucuronidase reporter gene using mature zygotic embryos of three genotypes of loblolly pine as explants.

Materials and methods

Plant materials

Mature seeds of three genotypes (E-Hb, E-Ma, and E-Mc) were collected from Shaoyang Seed Orchard, Hunan Province, China in October 1996, and stored in plastic bags at 4 °C before they were used to tissue culture. Seeds were disinfected by immersion in 70% w/w ethanol alcohol for 30 s and in 0.1% mercuric chloride for 10-15 min, followed by four to five rinses in sterile distilled water. Mature zygotic embryos were aseptically removed from the megametophytes and placed horizontally on a solified callus induction medium in flasks or Petri dishes. Mature zygotic embryo explants were used to transformation experiments after cultured on pretreatment medium consisted of TE medium (Tang *et al.* 1998) supplemented with 10 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D), 4mg/L benzyladenine (BA), and 4mg/l kinetin for 1-3 weeks.

Agrobacterium strain and plasmid

Agrobacterium tumefaciens strain LBA4404 containing the binary plasmid pBI121 (Clontech) (Fig. 1) carrying neomycin phosphotransferase gene (nptII) which confers kanamycin resistance, and β-glucuronidase (GUS) under the control of the cauliflower mosaic virus 35S promoter and the terminator from nopaline synthase (nos) were used in the transformation experiments. *Agrobacterium tumefaciens* was grown overnight at 28 °C in liquid YEP medium (Sambrook *et al.* 1989) supplemented with 100 mg/L carbenicillin and 50 mg/L kanamycin. The overnight culture was used for transformation of mature zygotic embryos. Concentration of bacterium was determined in MILTON ROT spectronic 1201 at 600 nm.

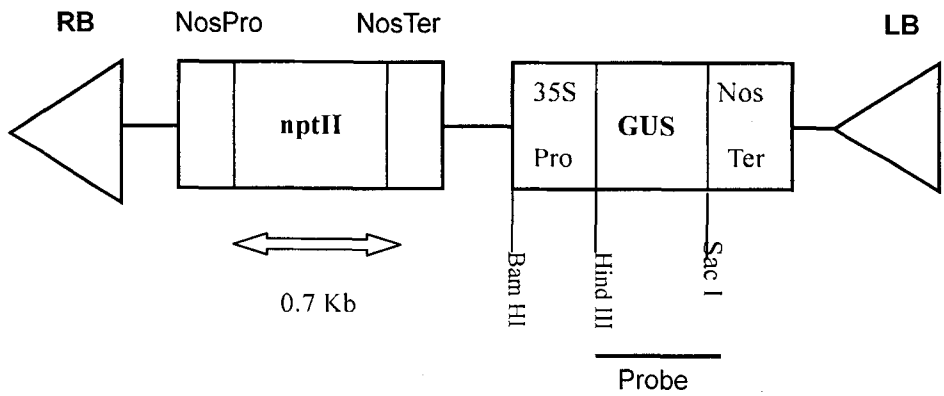

Fig. 1 T-DNA region of pBI121 plasmid, the 2kb BamHi-SacI fragment of pBI121 that was used as the DNA probe in the Southern blot analysis.

Note: RB--right border of T-DNA, LB--left border of T-DNA, NosPro-- promoter of the nopaline synthase gene, NosTer-- terminator of the nopaline synthase gene, 35Spro--the cauliflower mosaic virus 35S promoter, nptII--neomycin phosphotransferase gene, GUS--β-glucuronidase gene.

Co-cultivation and selection

After mature zygotic embryos were infected with bacterium cultures (OD600: 0.5-1.0) for 15-25 min, cocultivation was conducted at 25 °C for 3-5 d in the darkness on callus induction medium consisted of TE medium (Tang *et al.* 1998) supplemented with 10 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D), 4 mg/L benzyladenine (BA), 4 mg/L kinetin, and 50 (M acetosyringone. Co-cultivated mature zygotic embryos

were washed 4-5 times in sterile distilled water for reducing the *Agrobacterium* contamination, and were placed on sterile filter paper to remove excess liquid and transferred onto callus induction medium supplemented with 500 mg/L carbenicillin (for killing *Agrobacterium tumefaciens* LBA4404) for 1 week. Then cocultivated mature zygotic embryos were transferred onto callus induction medium supplemented with 500 mg/L carbenicillin (for killing *Agrobacterium tumefaciens* LBA4404) and 15 mg/L kanamycin (for killing non-transformed cells and tissues). After three weeks, mature zygotic embryos were transferred to fresh callus induction medium. Plant regeneration was carried out according to a procedure previously described (Tang *et al.* 1998).

Histochemical assays and fluorimetric analysis of GUS activity

Histochemical analysis of GUS expression was performed 24 h after cocultivation with *Agrobacterium tumefaciens*. Tissues were incubated in staining buffer (Jefferson *et al.* 1987) consisting of 100 mM sodium phosphate, 50 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid cyclohexylammonium salt, 0.1% β-mercaptoethanol, and 0.1% Triton X-100 (pH 7.2) at 37 °C for 16 h prior to observation. Stained plant materials were cleared with 70% ethanol for at least 5 times. A sample was scored as GUS positive if there was at least one discrete dark-blue region on the tissue. Fluorimetric assays were performed using a TK100 fluorimeter (Hoefer, San Francisco, Calif.). Total protein was extracted from plant tissue with extraction buffer (Jefferson *et al.* 1987) and was measured using the BioRad protein assay (according to BioRad instructions). Fluorimetry was according to the protocol by Jefferson *et al.* (1987), including the appropriate experimental controls.

PCR analysis

Loblolly pine genomic DNA was prepared by the method of Wagner *et al.* (1987). Primers used for amplification of insert DNA were the nptII forward primer 5'-ACAAACAGACAATCGGCTGC-3' and reverse primer 5'-AAGAACTCGTCAAGAAGGCG-3'. A total of 100-300 ng genomic DNA was used as template in a 50 μLPCR reaction mix containing 200 μM each of dATP, dCTP, dGTP, dTTP, 0.25 μM of each primer, 2U Taq DNA polymerase (Promega), 1.5 mM MgCl2, and 5 μL 10 × buffer. The reaction proceeded in a programmable Amplitron I Thermal Cycler for 30 cycles (denaturation at 94 °C for 40 s, annealing at 50 °C for 1 min, and extension at 72 °C for 1.5 min). PCR products were observed under UV after electrophoresis on a 0.8% agarose gel. Molecular markers are (DNA Hind III and 1Kb marker (Gibco-BRL).

Southern blot

Genomic DNA was isolated from control and transgenic plants according to the methods of Wagner *et al.* (1987). Thirty micrograms of DNA was digested overnight with the restriction enzyme EcoRI and Hind III (Boehringer Mannheim) at 37 °C, then electrophoresed on a 1×TAE, 0.8% agarose gel in TAE and denatured with 0.5 N NaOH, then transferred to a nylon membrane using alkali transfer buffer. The DNA fixed on membranes was hybridized (at 65 °C) with the uidA (GUS) probe (BamHI and SacI (Boehringer Mannheim) fragment of GUS gene), which was labeled with 32P-dCTP (Ready to Go Labeling Beads (Pharmacia)), according to standard protocols (Sambrook *et al.* 1989). Membranes were washed twice in 2×SSC, 0.1% SDS at 65 °C for 5 min each, once in 0.5 (SSC, 0.1 % SDS at 62 °C for 15 min, and once in 0.1×SSC, 0.1% SDS at room temperature for 30 min, and exposed to Kodak X-Omat-AR films at –80 °C for three days.

Results and discussion

Induction of transgenic callus

Before the transformation experiments, mature zygotic embryos were cultured on callus induction medium containing 0, 5, 10, 15, 20, 25, and 30mg/L kanamycin to identify the optimal concentration for selecting transformed mature zygotic embryos. Callus and adventitious bud formation were completely inhibited at 15mg/L kanamycin in the 4th week of culture (Fig. 2). One to three weeks after cocultivated mature zygotic embryos were transferred transferred onto callus induction medium supplemented with 500 mg/L carbenicillin and 15 mg/L kanamycin, mature zygotic embryos began to form calli. The frequency of transgenic calli increased during 3-8 weeks on fresh callus induction medium supplemented with 500mg/L carbenicillin and 15mg/L kanamycin. The highest frequency of transgenic callus formatiom was obtained on the 8th week. To improve the transformation efficiency, different concentrations of coniferyl alcohol were added to media during the period of cocultivation. Eight weeks after inoculation, the frequency of kanamycin-resistant callus formation was obviously improved (Fig. 3). The optimal concentration of coniferyl alcohol for improving the transformation efficiency (HB 18.4%, Ma 21.5%, and Mc 27.8%) was 100 μM. Two to three weeks after inoculation, callus was formed on cotyledons, hypocotyl, and radicles of embryos inoculated with bacteria. Proliferation of transgenic calli was achieved by subcultured this type of calli on fresh callus induction medium with selected abtibiotics.

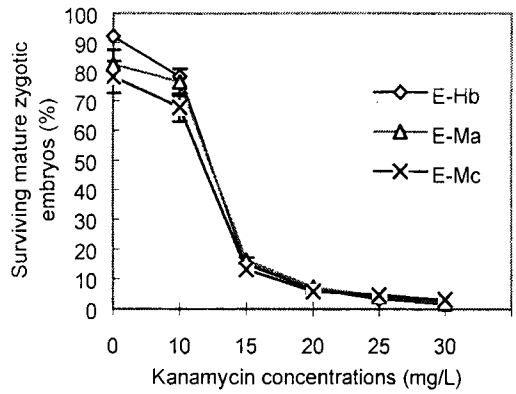

Fig. 2 Influence of kanamycin concentrations on the survival of mature zygotic embryos of three genotypes of loblolly pine.

(Each treatment was replicated three times, and each replicate consisted of 50-90 mature zygotic embryos. Errors represent the standard deviation around the mean).

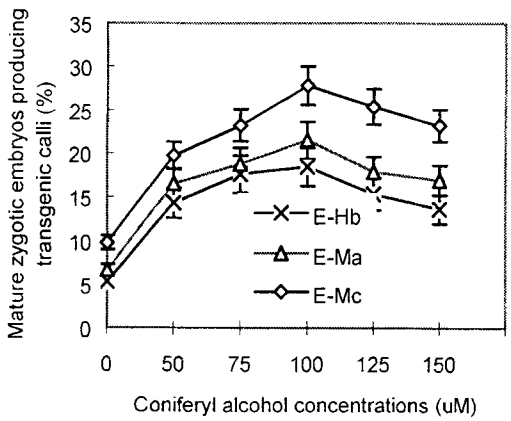

Fig. 3 Influence of coniferyl alcohol concentrations on kanamycin-resistant callus formation of mature zygotic embryos of three genotypes of loblolly pine

(Each treatment was replicated three times, and each replicate consisted of 50-90 mature zygotic embryos. Errors represent the standard deviation around the mean).

Expression of GUS gene in transgenic tissues.

Genotype was a significant factor in frequency of GUS expression of infected mature zygotic embryos. The frequency of GUS expression and the number of blue GUS spots per embryo varied among genotype. The highest frequency of GUS expressing embryos was obtained from genotype E-Mc (Hb 18.1%, Ma 21.9%, and Mc 27.1%). In a culture period of five weeks after infection (Fig. 4), mature zygotic embryos of genotype E-Mc had the highest mean number of blue GUS spots per embryo (21.9 blue GUS spots per embryo) Transient GUS expression was observed mostly in cotyledons and hypocotyls of the mature zygotic embryos of genotype E-Hb, and in radicles of the mature zygotic embryos of genotype E-Ma and E-Mc (Fig. 5a). Organogenic callus with GUS expression was obtained from these three genotypes tested. Accumulation of GUS enzyme was not detected in uninoculated control embryos. Longer cocultivatrion period, up to 2 weeks, resulted in more embryos showing positive GUS expression, but also led to a considerable decrease in embryo survival rate. GUS expression were observed in adventitious buds regenerated from mature zygotic embryo cultures of genotype E-Hb, E-Ma, and E-Mc. Differences of foreign gene expression among conifer species and genotypes were observed in spruce and larch (Ellis *et al.* 1989; McAfee *et al.* 1993; Diner *et al.* 1987; Clapham *et al.* 1986). To improve the transformation efficiency, coniferyl alcohol was added to media during the period of cocultivation. The results show that the frequency of GUS expressing tissues and kanamycin-resistant callus (Fig. 5b) formation was obviously improved. That the addition of virulence inducers increased transformation events was also reported in hybrid larch (Levee *et al.* 1997) and Norway spruce (Wenck *et al.* 1999), as well as other plant species (Godwin *et al.* 1991; James *et al.* 1993; Sheikholeslam *et al.* 1987; Stachel *et al.* 1985). To confirm that the positive X-Gluc in mature zygotic embryos was not due to the presence of viable *Agrobacterium tumefaciens* in the explants, homogenized callus of the GUS+ and GUS- was plated on YEP medium. After 1 week of inoculation at 28 °C, no bacterium growth was visible from any of putatively transformed mature zygotic embryos.

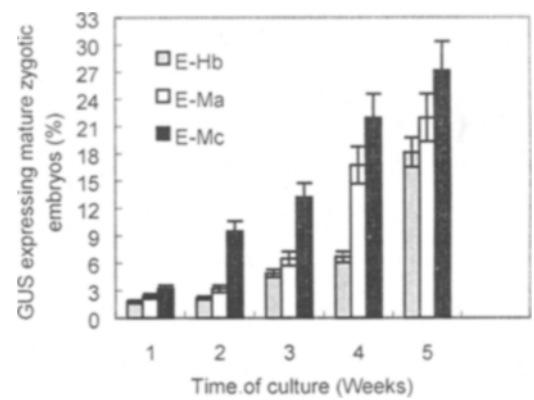

Fig. 4 Influence of different genotypes of loblolly pine on the induction frequency (%) of GUS expressing mature zygotic embryos

(Each treatment was replicated three times, and each replicate consisted of 150-200 mature zygotic embryos. Errors represent the standard deviation around the mean).

Differentiation of transgenic shoots and plant regeneration

Nine to twelve weeks after kanamycin-resistant calli were transferred to differentiation medium for regenerating adventitious buds, adventitious buds were formed on the surface of kanamycin resistant calli (Fig. 5c). The frequency of adventitious bud formation was 7.1%-16.9% on the differentiation medium supplemented with BA and IBA in the 12th week of culture (Fig. 6). Both rooting of adventitious buds and acclimatization of regenerated plantlets were carried out according to a procedure previously described (Tang *et al.* 1998). Rooting frequency 4.3%-16.9% was observed and both growth and phenotype of regenerated plantlets appeared similar to the untreated control. Nineteen regenerated plantlets (Hb 5, Ma 7, and Mc 7) from three genotypes of loblolly pine were transferred from culture flasks into a perlite : peatmoss : vermiculite (1:1:1) soil mixture, and seven acclimatized plantlets (Hb 2, Ma 2, Mc 3) were successfully established in the field (Fig. 5d). Fluorometric assay of GUS activity in transgenic tissues and regenerated plantlets of different genotypes of loblolly pine show that the highest GUS activity was obtained from transgenic regenerated plantlets of three genotypes (Fig. 7).

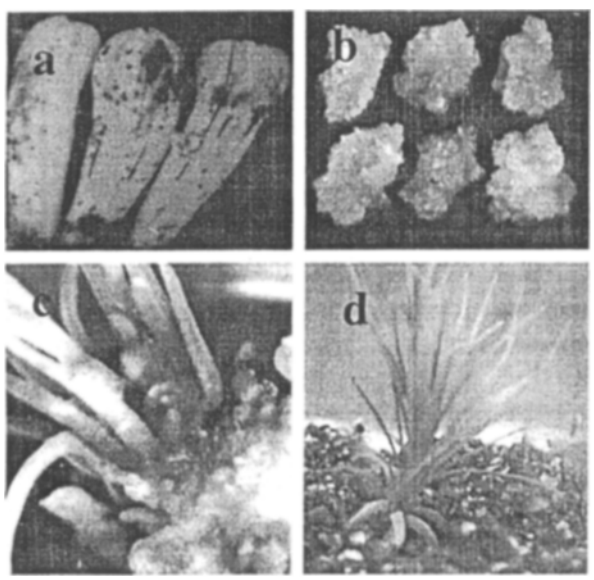

Fig. 5 *Agrobacterium tumefaciens*-mediated transformation and transgenic plant regeneration in loblolly pine.

a--GUS expression on cotyledons and hypocotyl (bar = 0.3 cm);
b--Kanamycin-resistant calli derived from cotyledons (bar = 0.5 cm),
c-- Kanamycin-resistant adventitious shoots (bar = 0.8 cm).
d--Transgenic regenerated plantlet established in soil (bar = 1 cm).

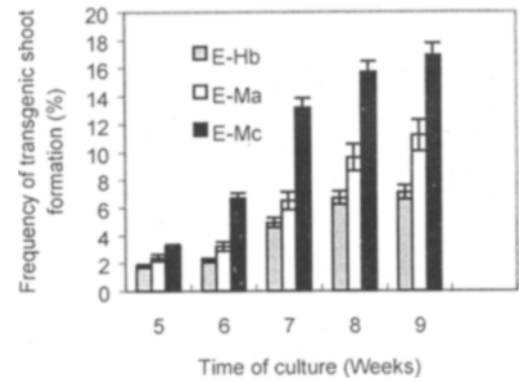

Fig. 6 Influence of different genotypes of loblolly pine on the regeneration frequency (%) of transgenic adventitious buds

(Each treatment was replicated four times, and each replicate consisted of 90-150 callus tissues. Errors represent the standard deviation around the mean).

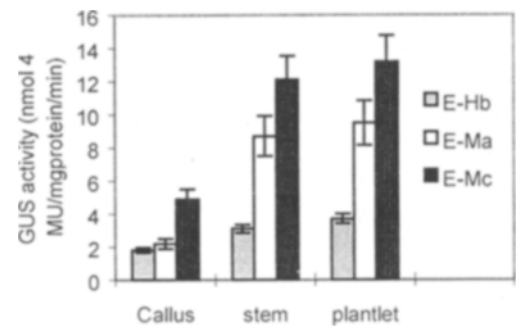

Fig. 7 Fluorometric assay of GUS activity in transgenic tissues and regenerated plantlets of different genotypes of loblolly pine

(Each treatment was replicated three times, and each replicate consisted of 300-500 mg tissues. Errors represent the standard deviation around the mean).

PCR analysis of the transformants

Loblolly pine genomic DNA was prepared by the method of Wagner *et al.* (1987). PCR analysis was carried out as a rapid identification for the presence of the insert DNA in kanamycin resistant calli from three genotypes of loblolly pine. The expected 717bp band was amplified in the kanamycin resistant and GUS expressed calli. No insert DNA band was amplified in the nontransformed calli. Some of the regenerated plantlets derived from kanamycin- resistant calli of three genotypes of loblolly pine were also checked by PCR (Fig. 8). All of the kanamycin-resistant plantlets tested show the 717 bp band. Thus, it confirmed that these regenerated plantlets from inoculation were transformants.

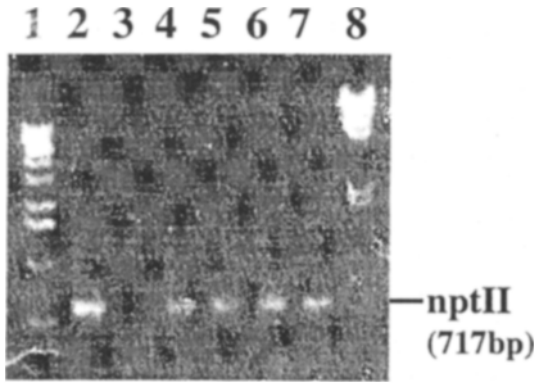

Fig. 8 PCR analysis of DNA isolated from putative transgenic plantlets

(Lane 1--1kb molecular markers (Gibco-BRL), lane 2--pBI121 plasmid control, lane 3--non-transgenic regenerated plantlet control, lanes 4 through 7-- transgenic regenerated plantlets of E Hb, E-Ma, and E-Mc, and transgenic shoot of E-Mc, respectively, lane 8--λDNA *Hind* III molecular markers (Gibco-BRL)

Southern blot

Trangenic plantlets from independent transformation events of three genotypes were analyzed by Southern hybridisation (Fig. 9). Genomic DNA was isolated from control and transgenic plants according to the methods of Wagner *et al.* (1987). Thirty micrograms of DNA was digested overnight with the restriction enzyme EcoRI or Hind III (Boehringer Mannheim) at 37 °C and was used in Southern hybridisation experoments. No bands were detected in nontransformed control plants, whereas bands were observed (Fig. 9) in transgenic plants. These results confirm the presence of foreign genes integrated into the *Pinus taeda* genome. The Southern results of regenerated transformed plants showed that one and two bands varied in size, respectively, which represented junctions between T-DNA and adjacent plant DNA. These findings show that these are transformants in which the insert DNA containing GUS gene has integrated at one and two sites, respectively, in the plant genome. The integration of tandem or multimer copies in regenerated transformed plants was also observed in *Pinus radiata* via particle bombardment (Walter *et al.* 1998).

The data on *Agrobacterium tumefaciens*-mediated transformation of three genotypes of loblolly pine presented here show a useful procedure for the regeneration of transgenic loblolly pine plantlets. This is the first report on transgenic plantlet regeneration through *Agrobacterium tumefaciens*-mediated transformation in loblolly pine. In the present investigation, we found that the efficiency of the transformation events was dependent on the genotypes infected and on the organs and tissues infected. High transformation rate and the intensity of GUS expression were observed in genotype E-Mc and to a lesser extent in genotype E-Hb, and E-Ma.

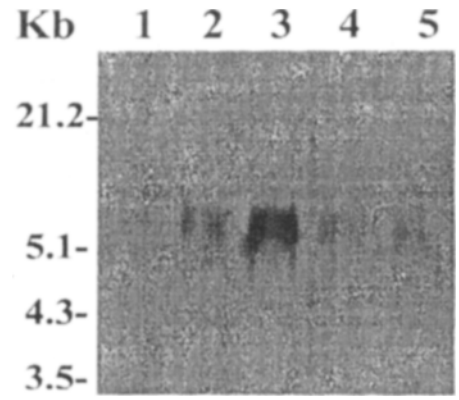

Fig. 9 Southern blot analysis of transgenic loblolly pine plants.

DNA was digested overnight with the restriction enzyme EcoRI and Hind III (Boehringer Mannheim), was hybridized (at 65°C) with the GUS probe (BamHI and SacI fragment of GUS gene), which was labeled with ^{32}P-dCTP (Ready to Go Labeling Beads (Pharmacia)), lane 1 is DNA from un-transformed plant of genotype E-Mc (30 μg), lane 2-4 DNA from transgenic plants of genotype E-Hb, E-Ma, and E-Mc, respectively (30 μg), and lane 5 is plasmid DNA of pBI121 (5pg).

Among different tissues, high GUS expression was observed in cotyledons, not in hypocotyl and radicle. This result is different from a previous report on the transformation of *Pinus halepensis* (Tzfira *et al.* 1996). It is probably due to more rapid formation of callus from cotyledons of mature zygotic embryos, compared to hypocotyls and radicles in our culture system. The induction frequency of calli is dependent up on the genotypes on the presence of kanamycin. The addition of 100 (M coniferyl alcohol during the cocultivation step increased the induction frequency of kanamycin resistant calli and Gus expressing tissues (Fig.3). This result is similar to the report of Levee *et al* (1997). Coniferyl alcohol is a phenolic compound that is released by the wounded plant cells and is a virulence inducer similar to acetosyringone (Stachel *et al.* 1985) and syringaldehyde. It plays an important role in the natural infection of plants by *Agrobacterium tumefaciens* because it activates the virulence genes of the Ti pasmid and initiates the transfer of the T-DNA region into a plant. A positive effect of phenolic compounds on *Agrobacterium tumefaciens*-mediated transformation has been demonstrated in many plant species, such as *Arabidopsis thaliana* (Sheikhloeslam *et al.* 1987), *Nicotina ta-*

bacum (Godwin *et al.* 1991), *Cucumis sativus* (Sarmento *et al.* 1992), and *Malus pumila* (James *et al.* 1993). Our results suggest that the effect of coniferyl alcohol on the *Agrobacterium tumefaciens*-mediated transformation of mature zygotic embryos varies among genotypes. The transformation procedure described in this paper show that it will be possible to establish a very useful *Agrobacterium tumefaciens*-mediated protocol for gene tagging and stable integration of genes into loblolly pine cultures which are capable of plant regeneration, and that this transformation system is probably useful for future studies on transferring economically important genes to loblolly pine.

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References

Bekkaoui, F., Pilon, M., Laine, E., Raju, D.S.S., Crosby, W.L., Dunstan, D.I. 1988. Transient gene expression in electroporated *Picea glauca* protoplasts [J]. Plant Cell Rep., **7**: 481-484.

Bergmann, B.A, Stomp, A.M. 1992. Effect of host plant genotype and growth rate on *Agrobacterium tumefaciens*-mediated gall formation in *Pinus radiata* [J]. Phytopathology, **82**: 1457-1462.

Bommineni, V.R, Chibbar, R.N, Dalta, RSS, Tsang, E.W.T. 1993. Transformation of white spruce (*Picea glauca*) somatic embryos by microprojectile bombardment [J]. Plant Cell Rep., **13**: 17-23.

Charset PJ, Michel, M, F. 1991, Basic of plant genetic engineering and its potential application to tree species [R]. Petawawa National Forestry Institute, Forestry Canada, Information Report PI-X-**104**: 48.

Charest, P.J, Devantier, Y., Lachance, D. 1996. Stable genetic transformation of Picea mariana (black spruce) via mocroprojectile bombardment [J]. In Vitro Cell Dev Biol., **32:** 91-99.

Clapha, D.H, Ekberg I. 1986. Induction of tumours by various strains of *Agrobacterium tumefaciens* on *Abies nordmanniana* and *Picea abies* [J]. Scand J For Res **1**: 435-437.

Diner, A.M, Karnosky, D.F. 1987. Differential responses of two conifers to in vitro inoculation with Agrobacterium rhizogenes [J]. Eur J For Path., **17**: 211-216.

Ellis, D.D, McCabe, D.E., Mcinnis, S., Ramachandran, R., Russell, D.R., Wallace, K.M., Martinell, B.J, Roberts, D.R., Raffa, K.F., McCown, B.H. 1993. Stable transformation of Picea glauca by particle acceleration [J]. Biotechnology, **11**: 84-89.

Ellis D., Roberts D., Sutton B., Lazaroff, W, webb D., and Flinn B. 1989. Transformation of white spruce and other conifer species by *Agrobacterium tumefaciens* [J]. Plant Cell Rep **8**: 16-20.

Godwin I, Gordon T, Ford-lloyd, B., Newbury, H.J. 1991. The effects of acetosyringone and pH on Agrobacterium-mediated transformation vary according to plant species [J]. Plant Cell Rep., **9**: 671-675.

Gupta, P.K, Dandekar. A.M., Durzan, D.J. 1988. Somatic proembryo formation and transient expression of a luciferase gene in Douglas fir and loblolly pine protoplasts [J]. Plant Sci., **58**: 85-92.

Gupta, P.K, Pullman, G., Timmis, R., Kreitinger, M., Carlson WC, Grob, J., Welty. E. 1993. Forestry in the 21st Century: The biotechnology of somatic embryogenesis [J]. Bio / Technology, **11**: 454-459.

Huang, Y., Diner, A.M, Karnosky, D.F. 1991. *Agrobacterium rhizogenes*-mediated genetic transformation and regeneration of a conifer: *Larix deciduas* [J]. In Vitro Cell Dev Biol., **27**: 201-207.

Humara, J.M, Lopez, M., Ordas R.J. 1999. *Agrobacterium rhizogenes*-mediated transformation of *Pinus pinea* L. cotyledons: an assessment of factors influencing the efficiency of uidA gene transfer [J]. Plant Cell Rep., **19**: 51-58.

James, C., Krattiger, A.F. 1996. Global review of the field testing and commercialisation of transgenic plants [R]. ISAAA Briefs No 1.

James, D.J, Uratsu, S., Cheng, J., Negri. P., Viss, P., Dandekar, A.M. 1993. Acetosyringone and osmoprotectants like betaine or proline synergistically enhance Agrobacterium-mediated transformation of apple [J]. Plant Cell Rep., **12**: 559-563.

Jefferson, R.A., Kavanagh, T.A., Bevan, M.W. 1987. GUS fusion: β-glucuronidase as a sensitive and versatile gene fusion marker in higher plants [J]. EMBO J., **6**: 3901-3907.

Klimaszewska, K., Devantier, Y., Lachance, D., Lelu, M.A., Charest, P.J. 1997. *Larix laricina* (tamarack): somatic embryogenesis and genetic transformation [J]. Can J For Res., **27**: 538-550.

Levee, V., Lelu, M.A., Jouanin, L., Cornu, D., Pilate, G. 1997. *Agrobacterium tumefaciens*-mediated transformation of hybrid larch (*Larix kaempferi* (*L. decidua*)) and transgenic plant regeneration [J]. Plant Cell Rep., **16**: 680-685.

McAfee, B.J, White, E.E., Pelcher, L.E., Lapp, M.S. 1993 Root induction in pine (*Pinus*) and larch (*Larix*) spp. Using *Agrobacterium rhizogenes* [J]. Plant Cell, Tiss Org Cult., **34**: 53-62.

Morris, J.W., Castle, L.A., Morris, R.O. 1989. Efficacy of different *Agrobacterium tumefaciens* strains in transformation of pinaceous gymnosperms [J]. Physiol. Mol Plant Patho., **34**: 451-461.

Robertson, D., Weissinger, A.K., Ackley, R., Glover, S., Sederoff, R.R. 1992. Genetic transformation of Norway spruce[*Picea abies* (L.) Karst] using somatic embryo explants by microjectile bombardment [J]. Plant Mol. Biol.,

19: 925-935.

Sambrook, J., Fritsch, E.F., Mamiatis, T. 1989. Molecular Cloning: a laboratory manual [M]. 2nd ed., Cold Spring Harbor, NY. USA.

Sarmento, G.G., Alpert, K., Tang, F.A., Punja, Z.K. 1992 Factors influencing *Agrobacterium tumefaciens*-mediated transformation and expression of kanamycin resistance in pickling cucumber [J]. Plant Cell, Tissue Organ Cult., **31**: 185-193.

Sederoff, R., Stomp, A.M., Chilton, W.S., Moore, L.W .1986. Gene transfer into loblolly pine by *Agrobacterium tumefaciens* [J]. Biotechnilogy, **4**: 647-649.

Sheikholeslam, S.N., Weeks, D.P. 1987. Acetosyringone promotes high frequency transformation of *Arabidopsis thaliana* explants by *Agrobacterium tumefaciens* [J]. Plant Mol Biol., **8**: 291-298.

Shin, D.I., Podila, G.K., Huang, Y., Karnosky, D.F 1994. Transgenic larch expressing genes for herbicide and insect resistance [J]. Can. J. For. Res., **24**: 2059-2067.

Stachel, S.E., Messens, E., Montague, M.V., Zambryski, P. 1985. Identification of the signal molecules produced by wounded plant cells that activate T-DNA transfer in *Agrobacterium tumefaciens* [J]. Nature, **318**: 624-629.

Stomp, A.M., Weissinger, A., Sederoff, R.R. 1991 Transient expression from microprojectile-mediated DNA transfer in *Pinus taeda* [J]. Plant Cell Rep., **10**: 187-190.

Stomp, A.M., Loopstra, C., Chilton, W.S., Sederoff, R.R., Moore, L.W. 1990. Extended host range of Agrobacterium tumefaciens in the genus *Pinus* [J]. Pant Physiol., **92**: 1226-1236.

Tang, W., Ouyang, F., Guo, Z.C. 1998. Plant regeneration through organogenesis from callus induced from mature zygotic embryos of loblolly pine [J]. Plant Cell Rep., **17**: 557-560.

Tzfira, T., Yarnitzky, O., Vainstein, A., Altman, A. 1996. *Agrobacterium rhizogenes*-mediated DNA transfer in *Pinus halepensis* Mill [J]. Plant Cell Rep., **16**: 26-31.

Wagner, D. B., Furnier, G. R., Saghai-Maroof, M.A., Williams, S.M., Dancik, B.W., Allard, R.W. 1987. Chloroplast DNA polymorphisms in lodgepole and jack pine and their hybrids [J]. Proc Natl Acad Sci USA **84**: 2097-2100.

Walter, C., Grace, L.J., Wagner, A., White, D.W.R, Walden A.R., Donaldson, S.S., Hinton, H., Gardner, R.C, Smith D.R. 1998. Stable transformation and regeneration of transgenic plants of *Pinus radiata* D.Don [J]. Plant Cell Rep., **17**: 460-469.

Walter, C., Grace, L., Donaldson, S.S., Moody, J., Gemmell, J.E., van der Maos, S., Kvaalen, H., Lonneborg, A. 1999 An efficient Biolistic transformation protocol for *Picea abies* embryogenic tissue and regeneration of transgenic plants [J]. Can. J. For Res.. **29**: 1539-1546.

Wenck, A.R., Quinn, M., Whetten, R.W., Pullman, G., Sederoff, R. 1999. High-efficiency *Agrobacterium*- mediated transformation of Norway spruce (*Picea abies*) and loblolly pine (*Pinus taeda*) [J]. Plant Mol. Biol., **39**: 407-416.